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Monosaccharide digitoxin derivative sensitize human non-small cell lung cancer cells to anoikis through Mcl-1 proteasomal degradation

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ABSTRACT

Advanced stage cancers acquire anoikis resistance which provides metastatic potential to invade and form tumors at distant sites. Suppression of anoikis resistance by novel molecular therapies would greatly benefit treatment strategies for metastatic cancers. Recently, digitoxin and several of its novel synthetic derivatives, such as α-L-rhamnose monosaccharide derivative (D6-MA), have been synthesized and studied for their profound anticancer activity in various cancer cell lines. In this study, we investigated the anoikis sensitizing effect of D6-MA compared with digitoxin to identify their anti-metastatic mechanism of action. D6-MA sensitized NSCLC H460 cells to detachment-induced apoptosis with significantly greater cytotoxicity (IC50 = 11.9 nM) than digitoxin (IC50 = 90.7 nM) by activating caspase-9. Screening of the Bcl-2 protein family revealed that degradation of anti-apoptotic Mcl-1 protein is a favorable target. Mcl-1 over-expression and knockdown studies in D6-MA and digitoxin exposed cells resulted in rescue and enhancement, respectively, indicating a facilitative role for decreased Mcl-1 expression in NSCLC anoikis. Transfection with mutant Mcl-1S159 attenuated detachment-induced cell death and correlated with a remaining of Mcl-1 level. Furthermore, D6-MA suppressed Mcl-1 expression via ubiquitin proteasomal degradation that is dependent on activation of glycogen synthase kinase (GSK)-3β signaling. In addition, D6-MA also targeted Mcl-1 degradation causing an increased anoikis in A549 lung cancer cells. Anoikis sensitizing effect on normal small airway epithelial cells was not observed indicating the specificity of D6-MA and digitoxin for NSCLC. These results identify a novel cardiac glycoside (CG) sensitizing anoikis mechanism and provide a promising anti-metastatic target for lung cancer therapy.

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1. Introduction

Late stage aggressive cancers undergo the phenomenon of metastasis, usually preceded by invasion of neighboring tissues. Metastasis involves cancer cells breaking free from the initial tumor mass, circulatory system transportation, reattachment at a target organ and prolonged growth, resulting in drastically elevated mortality rates [1–3]. Normal cells that detach from their extracellular matrix (ECM) quickly undergo anoikis, a programmed cell death via the mitochondrial apoptosis pathway [4,5]. Metastatic cancers, such as late stage III and stage IV lung cancer, resist anoikis via several different mechanisms including altered adhesion and growth factor signaling, epithelial to mesenchymal transition, and dysregulation of pro-apoptotic Bcl-2 proteins [6]. Metastatic lung tumors greatly reduce lung cancer survival rates by more than 40% compared to early stage tumors and cause death rates 2–4 times higher than all other cancers [7–9]. Development of novel, late stage cancer therapeutic strategies is clearly warranted. Given that 80–85% of lung cancers are non-small cell lung cancers (NSCLC),

Abbreviations: Bcl-2, B-cell lymphoma 2; Casp-3, caspase-3; CHX, cycloheximide; CG, cardiac glycoside; DMSO, dimethyl sulfoxide; D6-MA, α-L-rhamnose monosaccharide derivative; ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; FAK, focal adhesion kinase; GSK-3β, glycogen synthase kinase-3β; JNK, c-Jun amino-terminal kinase; MOA, mode of action; NSCLC, non-small cell lung cancer; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PI3K, phosphatidylinositol-3 kinase; TDZD, thiadiazolidinones.

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development of therapeutic strategies to sensitize aggressive NSCLC to anoikis would greatly improve malignant chemotherapy strategies and lung cancer patient prognosis.

Epidemiologic evidence suggests that congestive heart failure patients on cardiac glycoside (CG) therapy (i.e. digitalis 10–40 nM) exhibit reduced breast and leukemia cancer diagnoses and breast cancer reoccurrence [10–12]. Initial consensus on CG's anti-cancer mechanism of action (MOA) was that CGs act via direct binding to the α subunit of the Na^+/K^+ ATPase causing inotropic inhibition, internal Ca^+ ion release, thereby resulting in apoptosis initiation. More recent evidence suggests that Na^+/K^+ ATPase with other membrane-associated signaling proteins act as a 'signalosome' complex [13,14] that activates several signaling pathways resulting in in vitro anti-neoplastic activity [15,16]. Although cancer cells exhibit greater CG sensitivity than non-malignant cells, safety concerns with their clinical use remain due to cardiotoxicity, narrow therapeutic window and protein synthesis inhibition [17–19]. Nevertheless, low reoccurrence rate and in vitro cancer cell apoptotic sensitivity to CGs suggests that cancer cells may exhibit anoikis sensitivity to CG therapy. Surprisingly, little research effort has focused on CG-induced anoikis in cancer cells given the large volume of literature on apoptotic effect. By investigating the ability of CG's to induce both apoptosis and anoikis, researchers can identify whether CGs can therapeutically target primary tumors and/or their metastatic ability. Oleandrin, ouabain and UNBS1450 exhibit ability to sensitize cells to detachment-associated cell death via programmed cell death, autophagy and cellular swelling [20,21]. Mcl-1 expression, an anti-apoptotic Bcl-2 family protein, was recently described as a key anoikis resistance mediator and may serve as a CG target for cancer therapy [22–24].

Concerns with CG cardiotoxicity and a clear MOA have restricted their clinical use and anti-neoplastic research efforts. Recent advances, however, in synthetic carbohydrate chemistry and testing low dose effects (<50 nM) in vitro have further advanced CGs for anti-neoplastic therapy. Novel digitoxin derivative syntheses via Pd-catalyzed glycosylation [25,26] and in vitro cytotoxic screening using NCI's multiple attached cancer cell lines, including NSCLC cells [27,28], identified several monosaccharide digitoxin analogs displaying greater cytotoxic potency than digitoxin. The corresponding anti-neoplastic effects were identified as apoptotic cell death via caspase-9 intrinsic apoptotic pathway and G2/M arrest in attached NSCLC cells [27,29].

To further expand and identify digitoxin monosaccharide derivatives' anti-cancer modes of action, this study's primary objectives were to (a) compare α-L-rhamnose monosaccharide derivative (D6-MA) to digitoxin for their ability to sensitize NSCLC cells to anoikis and (b) determine the underlying anoikis sensitization mechanism. Our hypotheses stated that D6-MA would exhibit greater potency than digitoxin in initiating NSCLC anoikis via caspase-9 activation due to altered Mcl-1 expression. Identification of a NSCLC anoikis sensitization signaling mechanism for digitoxin-based derivatives will enhance understanding of CG apoptotic MOA and lend insight into whether CG reduce rate of cancer reoccurrence by inhibiting metastasis.

2. Methods

2.1. Cell culture and reagents

Human non-small cell lung cancer NCI-H460 and lung carcinoma A549 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Non-tumorigenic, human small airway epithelial cells immortalized with human telomerase (SAEC) were a kind gift from Dr. Tom Hei (Columbia University Medical Center). H460 cells were cultured in RPMI 1640 medium while A549 cells were cultured in DMEM medium (Sigma, St. Louis MO). All culture media contained 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin/streptomycin. SAECs were cultured in SABM medium supplemented with SAGM growth factor BulletKit (Lonza, Allendale, NJ) following manufacturer's instructions. Cells were cultured in a humid, 5% CO_2 environment at 37 °C. Hoechst 33342, cisplatin, cycloheximide, and poly(2-hydroxyethyl-methacrylate; poly-HEMA) were obtained from Sigma. MG132 was obtained from Calbiochem (San Diego, CA). Antibodies for poly-ADP-ribose polymerase (PARP), caspase-8, caspase-9, Mcl-1, phosphorylate Mcl-1, Bcl-2, Bax, Bim, Bid, β-actin, and peroxidase-labeled secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Ubiquitin antibody and protein-G agarose were obtained from Abcam Inc. (Cambridge, MA). Caspase-8 inhibitor (z-IETD-FMK), caspase-9 inhibitor (z-LEHD-FMK), pan-caspase inhibitor (z-VAD-FMK), and FasL were obtained from Alexis Biochemicals (San Diego, CA). Caspase-3 activity kit was purchased from Cell Signaling Technology, Inc. Caspase-8 and caspase 9 assay kits were obtained from BioVision (Milpitas, CA).

Digitoxin (>99% pure) was acquired from Sigma Chemicals (St. Louis, MO). D6-MA, and α-L-rhamnose monosaccharide digitoxin derivative (>98% pure) was synthesized using a previously described de novo Pd-catalyzed glycosylation method for unique carbohydrate synthesis [25,26]. Briefly, acetyl furan underwent asymmetric reduction, oxidative rearrangement and *tert*-butoxycarbonyl protection to acquire the correct glycosyl donor. The resulting α-L-pyranose was coupled to digitoxigenin, the acid-cleaved aglycone moiety of digitoxin, via Pd-catalyzed glycosylation followed by Luche reduction to acquire D6-MA. All D6-MA was purified using SiO_2 gel flash chromatography, recrystallized in either methyl chloride or ethyl chloride in hexane and dried under vacuum. Sample purity was determined via ^1H and ^{13}C NMR as well as melting point and optical rotation. The chemical structure was shown in Fig. 1A. Both compounds were diluted in sterile DMSO to 2 mM stock and serially diluted in DMSO to reach exposure doses.

2.2. Anoikis sensitivity determination

Anoikis sensitization assays on H460 cells were performed in 12-well plates coated with poly-HEMA to keep cells from attaching to well bottom. A 6 mg/mL poly-HEMA solution was prepared with warm 95% ethanol, pipetted into each well and allowed to evaporate overnight. Sub-confluent H460 cells were then PBS washed, 0.05% trypsinized, suspended in 1% FBS and diluted to 1×10^5 cells/mL in microfuge tubes. Cells were exposed to 0–500 nM by pipetting diluted compound (DMSO < 0.1% v/v) to each tube, triturated and seeded to each well. Following a 24 and 48 h exposure, 10 μM Hoechst 33342 and 5 μg/mL propidium iodide (PI) dissolved in PBS were added to each well and incubated for 30 min. Stained cells were immediately photographed using a Leica DFC 490 digital camera mounted on a Leica DMIL inverse compound microscope at 400× magnification. At least three replicates per dose per compound were run in each experiment which 3–5 experiments were performed. Percentage of cells displaying condensed chromatin and/or fragmented apoptotic nuclei and necrotic cell death were determined from 5 replicate photos of each experimental replicate. Cells displaying PI-stained fragmented nuclei were considered late-stage apoptotic nuclei. A minimum of 1000 cells were counted per treatment. IC_{50} analyses were conducted in GraphPad Prism 5 (La Jolla, CA).

2.3. Caspase activity determination

Caspase 3 activity was measured using Ac-DEVD-AMC caspase 3 activity assay kit (Cell Signaling Technology, Inc., Beverly, MA), and caspase 8 and 9 activations were using IETD-AFC caspase 8 and LEHD-AFC caspase 9 assay kits (BioVision, Milpitas, CA) following

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